Title: Identifying the Enzymes Responsible for Reduction of Doxorubicin to its Cardiotoxic Metabolite Doxorubicinol using a Novel Immunoclearing Approach.

Abstract approved:

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Gary F. Merrill

Doxorubicin is a widely used cancer therapeutic, but its effectiveness is limited by cardiotoxic side effects. Evidence suggests cardiotoxicity is due not to doxorubicin, but rather its metabolite, doxorubicinol. Identification of the enzymes responsible for doxorubicinol formation is important in developing strategies to prevent cardiotoxicity. In this study, the contributions of three murine candidate enzymes to doxorubicinol formation were evaluated: carbonyl reductase 1 (Cbr1), carbonyl reductase 3 (Cbr3), and thioredoxin reductase 1 (Tr1). Analyses with purified proteins revealed that all three enzymes catalyzed doxorubicin-dependent NADPH oxidation, but only Cbr1 and Cbr3 catalyzed doxorubicinol formation. Doxorubicin-dependent NADPH oxidation by Tr1 was likely due to redox cycling. Subcellular fractionation results showed that doxorubicin-dependent redox cycling activity was primarily microsomal, whereas doxorubicinol-forming activity was exclusively cytosolic, as were all three enzymes. An immunoclearing approach was used to assess the contributions of the three enzymes to doxorubicinol formation in
the complex milieu of the cytosol. Immunoclearing Cbr1 eliminated 25% of the total
doxorubicinol-forming activity in cytosol, but immunoclearing Cbr3 had no effect,
even in Tr1 null livers that overexpressed Cbr3. The immunoclearing results
constituted strong evidence that Cbr1 contributed to doxorubicinol formation in
mouse liver, but that enzymes other than Cbr1 also played a role, a conclusion
supported by ammonium sulfate fractionation results which showed that
doxorubicinol-forming activity was found in fractions that contained little Cbr1. In
conclusion, the results show that Cbr1 accounts for 25% of the doxorubicinol-forming
activity in mouse liver cytosol but that the majority of the doxorubicinol-forming
activity remains unidentified.
Identifying the Enzymes Responsible for Reduction of Doxorubicin to its Cardiotoxic Metabolite Doxorubicinol using a Novel Immunoclearing Approach.

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Daniel Breysse, Author
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Introduction</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Doxorubicin Cardiotoxicity</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Carbonyl Reductase 1</td>
<td>8</td>
</tr>
<tr>
<td>1.3 Carbonyl Reductase 3</td>
<td>10</td>
</tr>
<tr>
<td>1.4 Aldo-keto Reductase</td>
<td>11</td>
</tr>
<tr>
<td>1.5 Thioredoxin Reductase 1</td>
<td>12</td>
</tr>
<tr>
<td>1.6 Experimental Approach</td>
<td>12</td>
</tr>
<tr>
<td>2 Material and Methods</td>
<td>14</td>
</tr>
<tr>
<td>2.1 Mouse Liver Fractionation</td>
<td>14</td>
</tr>
<tr>
<td>2.2 Immunological Procedures</td>
<td>15</td>
</tr>
<tr>
<td>2.3 Enzyme Assays</td>
<td>19</td>
</tr>
<tr>
<td>3 Results</td>
<td>22</td>
</tr>
<tr>
<td>3.1 Dox-dependent NADPH oxidation and NADPH-dependent Doxol formation by purified Cbr1, Cbr3 and Tr1</td>
<td>22</td>
</tr>
<tr>
<td>3.2 Doxorubicin-dependent redox cycling catalyzed by Tr1</td>
<td>29</td>
</tr>
<tr>
<td>3.3 Subcellular localization of Dox-metabolizing activities in liver</td>
<td>29</td>
</tr>
<tr>
<td>3.4 Quantification of Cbr1 and Cbr3 protein in wild type and gclm-null mouse liver cytosol</td>
<td>32</td>
</tr>
<tr>
<td>3.5 Effect of immunoclearing Cbr1 on Doxol-forming activity in mouse liver cytosol</td>
<td>34</td>
</tr>
<tr>
<td>3.6 Effect of immunoclearing Cbr3 and Tr1 on Dox-dependent NADPH oxidation and Doxol-forming activity in mouse liver cytosol</td>
<td>37</td>
</tr>
<tr>
<td>3.7 Doxol formation in txnrd1-null mouse liver cytosol</td>
<td>39</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS (Continued)

3.8 Ammonium sulfate fractionation of Doxol-forming activity in liver cytosol ................................................................. 42

4 Discussion ................................................................................. 44

5 Conclusion ................................................................................ 48

6 Bibliography ............................................................................... 51

7 Appendix .................................................................................... 58

   A. Dox-dependent NADPH oxidation kinetics data ...................... 58

   B. NADPH-dependent Doxol formation kinetics data ................. 61

   C. Abbreviations ......................................................................... 64
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>The four most common anthracycline chemotherapeutics</td>
</tr>
<tr>
<td>2.</td>
<td>Redox cycling of Dox</td>
</tr>
<tr>
<td>3.</td>
<td>Conversion of Dox to its cardiotoxic metabolite Doxol</td>
</tr>
<tr>
<td>4.</td>
<td>Preparation of affinity-purified Cbr3-specific antibody</td>
</tr>
<tr>
<td>5.</td>
<td>Dox-dependent NADPH oxidation by purified recombinant Cbr1, Cbr3, and Tr1</td>
</tr>
<tr>
<td>6.</td>
<td>Representative LC-MS/MS chromatograms of Dox and Doxol reference standards</td>
</tr>
<tr>
<td>7.</td>
<td>NADPH-dependent Doxol formation by purified recombinant Cbr1, Cbr3, and Tr1</td>
</tr>
<tr>
<td>8.</td>
<td>H$_2$O$_2$ formation by Cbr1 and Tr1</td>
</tr>
<tr>
<td>9.</td>
<td>Subcellular localization of Cbr1, Cbr3 and Tr1 protein; Dox-dependent NADPH oxidation activity; NADPH-dependent Doxol-forming activity; and H$_2$O$_2$ formation in mouse liver</td>
</tr>
<tr>
<td>10.</td>
<td>Relative levels of Cbr1 and Cbr3 in wild type and gclm-null mouse liver cytosol</td>
</tr>
<tr>
<td>11.</td>
<td>Immunoclearing of Cbr1 and its effect on Doxol-forming activity in mouse liver cytosol</td>
</tr>
<tr>
<td>12.</td>
<td>Immunoclearing Cbr3 and Tr1 from mouse liver cytosols and effect on Dox-dependent NADPH oxidation and NADPH-dependent Doxol formation</td>
</tr>
<tr>
<td>13.</td>
<td>Cbr1 and Cbr3 levels and Doxol-forming ability of wt and txnrd1-null mouse liver cytosols</td>
</tr>
<tr>
<td>14.</td>
<td>Immunoclearing of Cbr1 and/or Cbr3, and its effect on Doxol-forming activity in txnrd1-null mouse liver cytosols that overexpress Cbr3</td>
</tr>
<tr>
<td>15.</td>
<td>Cbr1 levels and Doxol-forming activity in ammonium sulfate-fractionated mouse liver cytosol</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Kinetic parameters $K_m$, $k_{cat}$, and $k_{cat}/K_m$ for Dox-dependent NADPH oxidation by purified Cbr1, Cbr3, and Tr1</td>
<td>24</td>
</tr>
<tr>
<td>2. Kinetic parameters $K_m$, $k_{cat}$, and $k_{cat}/K_m$ for NADPH-dependent Doxol formation by purified Cbr1 and Cbr3, as well as $K_m$ and $V_{max}$ for NADPH-dependent Doxol formation by mouse liver cytosol</td>
<td>28</td>
</tr>
</tbody>
</table>
# LIST OF APPENDIX FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.1</td>
<td>Kinetic analysis of Dox-dependent NADPH oxidation by Cbr1</td>
<td>58</td>
</tr>
<tr>
<td>A.2</td>
<td>Kinetic analysis of Dox-dependent NADPH oxidation by Cbr3</td>
<td>59</td>
</tr>
<tr>
<td>A.3</td>
<td>Kinetic analysis of Dox-dependent NADPH oxidation by Tr1</td>
<td>60</td>
</tr>
<tr>
<td>B.1</td>
<td>Kinetic analysis of NADPH-dependent Doxol formation by Cbr1</td>
<td>61</td>
</tr>
<tr>
<td>B.2</td>
<td>Kinetic analysis of NADPH-dependent Doxol formation by Cbr3</td>
<td>62</td>
</tr>
<tr>
<td>B.3</td>
<td>Kinetic analysis of NADPH-dependent Doxol formation by mouse liver Cytosol</td>
<td>63</td>
</tr>
</tbody>
</table>
1.1: Doxorubicin Cardiotoxicity

Doxorubicin (Dox), also known by the trade name Adriamycin, is a chemotherapeutic used in the treatment of a broad variety of cancers. Dox is a member of the anthracycline class of chemotherapeutics, which were first isolated from *Streptomyces peucetius* as antibiotic compounds in 1964 (Dimarco et al., 1964b; Dubost et al., 1964). Daunorubicin, the first anthracycline discovered, was quickly found to be a highly effective anti-neoplastic agent (Dimarco et al., 1964a). Soon after, Dox (daunorubicin hydroxylated at the carbon-14 position) was isolated from a mutant strain of *S. peucetius* (var. caesius) (Arcamone et al., 1969; Di Marco et al., 1969). Dox was found to have more anti-tumor activity than its precursor and quickly became a common component of many chemotherapeutic regimens. Since the discovery of these first anthracyclines, many analogs of Dox and daunorubicin have been created and screened as potential cancer therapeutics, several of which see regular use in cancer treatment including epirubicin (Bonfante et al., 1980) and idarubicin (Arcamone et al., 1976; Di Marco et al., 1977) most prominently (Fig. 1). Despite fifty years of anthracycline development, Dox has remained the most effective and widely used anthracycline anti-cancer agent in use today (Weiss, 1992).

Despite its effectiveness in treating cancer, usage of Dox is limited by cardiotoxic side effects, which manifest as congestive heart failure, either during treatment or years after completion of therapy (Kremer and Caron, 2004; Lipshultz et al., 2013; Singal and Iliskovic, 1998; Swain et al., 2003). The cardiotoxicity of Dox is a property shared to a greater or lesser extent by all anthracycline drugs and is both...
Figure 1: The four most common anthracycline chemotherapeutics
Unique chemical features that differ between the structures are highlighted with arrows.
cumulative and dose-dependent. The maximum lifetime cumulative dosage of Dox is typically limited to 400-450 mg/m² to minimize the risk of cardiac dysfunction (Floyd et al., 2005; McGowan et al., 2017). One study of breast and lung cancer patients found that 26% of patients who received a cumulative dose of 550 mg/m² and 48% of those who received 700 mg/m² developed Dox-induced congestive heart failure (Swain et al., 2003). The cardiotoxic side effects caused by Dox are particularly deadly; patients who developed cardiomyopathies following Dox treatment experienced a mortality rate of 50% after two years (Felker et al., 2000). The dangerous cardiotoxicity combined with its striking red coloration have led to Dox colloquially being referred to as “red death” or “red devil”.

Studies into Dox-induced cardiotoxicity have explored how many of the same mechanisms which make Dox an effective antineoplastic agent may also be causing its cardiotoxicity. This is a difficult undertaking, as the mechanism of action for Dox is complex, multifactorial, and not well understood, even more than half a century after its initial discovery. Two important factors believed to contribute to the anti-tumor properties of Dox are the inhibition of Topoisomerase II (TopoII) (Marinello et al., 2018) and creation of reactive oxygen species (ROS) by redox cycling of Dox (Doroshow, 1983; Graham et al., 1987; Xu et al., 2001; Zhu et al., 2016). Both factors have been linked to Dox-induced cardiotoxicity.

TopoII is responsible for relieving topological tension in DNA by causing a double strand break, then reshaping the double helix and repairing the break. There are two subclasses of TopoII: TopoIIα and TopoIIβ (Wang, 2002). The major difference between the two lies in how they are regulated. TopoIIα is highly
expressed in actively replicating cells, while TopoIIβ is constitutively expressed in quiescent cells such as cardiomyocytes (McGowan et al., 2017; Turley et al., 1997; Zhang et al., 2012). As a TopoIIα poison, Dox binds to the TopoIIα-DNA complex and prevents the reformation of DNA following the double-strand break created as part of the normal function of TopoII, causing extensive DNA damage in replicating cells and inducing apoptosis (Marinello et al., 2018). However, binding of Dox with TopoIIβ appears to cause mitochondrial dysfunction which may be related to cardiotoxicity (McGowan et al., 2017). Mice with a cardiomyocyte-specific deletion of the gene encoding TopoII β were protected against Dox-induced cardiotoxicity, suggesting cardiotoxicity is the result of interaction between Dox and TopoIIβ (Zhang et al., 2012).

The formation of ROS by Dox is believed to be caused by redox cycling. During this process, an electron equivalent from NADPH is transferred to the quinone moiety of Dox to form a semiquinone, but is subsequently transferred to another entity, presumably solvent oxygen, which results in regeneration of Dox and the accumulation of superoxide and H₂O₂ (Fig. 2). Redox cycling is known to be catalyzed by a number of entities, including cytochrome p450, xanthine oxidase, NADH dehydrogenase, and thioredoxin reductase (Doroshow, 1983; Gewirtz, 1999; Graham et al., 1987; Hopkins, 2016; Ravi and Das, 2004; Zhu et al., 2016). As the heart is particularly susceptible to oxidative stress (Bains et al., 2009; Zhang et al., 2001), the formation of ROS by the redox cycling of Dox is a tempting explanation for Dox-induced cardiotoxicity (Doroshow et al., 1980; Octavio et al., 2012; Xu et al., 2001; Zhu et al., 2016). However, attempts to ameliorate or prevent Dox-induced
Figure 2: Redox cycling of Dox
The reaction proceeds by the one electron reduction of Dox to a semiquinone radical, with the flavin group of the enzyme presumably holding the second enzyme equivalent from NADPH. The semiquinone decays back to Dox, generating a superoxide which can be readily converted into $\text{H}_2\text{O}_2$ spontaneously or via superoxide dismutase.
cardiotoxicity caused by ROS using free radical scavengers have been unsuccessful, suggesting that regardless of the degree to which ROS contributes to cardiotoxicity, treatment targeting this pathway may not be practical (Myers et al., 1983; Unverferth et al., 1985).

One other proposed mechanism for Dox-induced cardiotoxicity involves doxorubicinol (Doxol), a prominent Dox metabolite that is generated by reduction of Dox at the C-13 carbonyl position (Fig. 3) (Boucek et al., 1987; Joerger et al., 2005; Olson et al., 1988). Doxol has been shown to be significantly more cardiotoxic than Dox. Doxol is far more potent than Dox in inhibiting several cardiac ion transporters, including the sarcolemmal Na\(^+\)/K\(^+\) channel, F\(_0\)F\(_1\) mitochondrial proton pump, sarcoplasmic reticulum Ca\(^{2+}\) ATPase, and ryanodine receptor (Boucek et al., 1987; Dodd et al., 1993; Hanna et al., 2014; Olson et al., 1988). Additionally, in a study of muscle isolated from rabbit hearts, Doxol was more potent than Dox in reducing myocyte contractility and increasing resting muscle tension, indicators of systolic and diastolic dysfunction, respectively (Boucek et al., 1987; Olson et al., 1988). In contrast to its greater cardiotoxicity, Doxol has weaker anti-neoplastic activity compared to Dox (Bernardini et al., 1991; Chang et al., 1989; Heibein et al., 2012; Olson et al., 1988). This is critical, as it suggests that the anti-tumor properties of Dox are not inherently linked to its cardiotoxicity. Preventing the metabolism of Dox to Doxol could thus increase the safety of the drug while retaining its effectiveness.

Several enzymes have been implicated as playing a role in Dox metabolism, including carbonyl reductases 1 and 3 (Cbr1, Cbr3) (Kassner et al., 2008; Schaupp et al., 2015), and aldo-keto reductases 1A1, 1B1, 1B10, and 1C3 (Akr1A1, Akr1B1,
Figure 3: Conversion of Dox to its cardiotoxic metabolite Doxol
The reaction proceeds via the two-electron reduction of the C-13 carbonyl group of Dox to an alcohol.
Akr1B10, Akr1C3) (Bains et al., 2008; Jin and Penning, 2007; Kassner et al., 2008; Morikawa et al., 2015; Sonowal et al., 2017).

1.2: Carbonyl Reductase 1

Cbr1 is a cytosolic, NADPH-dependent, short-chain oxidoreductase, first purified from human brain tissue by its ability to reduce menadione and other quinone-containing molecules (Wermuth, 1981). Cbr1 reduces both endogenous and xenobiotic substrates. Endogenous substrates of Cbr1 include prostaglandins, steroids, and lipid aldehydes, while its xenobiotic substrates include aromatic aldehydes and ketones, quinones, and nicotine-derived nitrosamine ketone, a procarcinogen found in tobacco (Forrest and Gonzalez, 2000; Hoffmann and Maser, 2007; Oppermann, 2007; Wermuth, 1981; Wermuth et al., 1986). Most importantly for this study, Cbr1 is known to catalyze the reduction of Dox to Doxol in vitro and is believed to be a prominent Dox-reductase in vivo (Bains et al., 2009; Forrest and Gonzalez, 2000; Kassner et al., 2008). Pharmacological studies suggesting Cbr1 is the primary Dox reductase focus on hydroxy-PP, a small molecule identified in a screen for specific Cbr1 inhibitors (Tanaka et al., 2005). Hydroxy-PP inhibited Doxol formation by liver cytosol with an inhibition constant of 9.9 µM, matching the K_i for hydroxy-PP inhibition of Doxol formation by purified Cbr1 (Kassner et al., 2008). Co-administration of hydroxy-PP with Dox in a rat model of breast cancer also attenuated cardiotoxicity and increased tumor sensitivity to Dox treatment (Jo et al., 2017). Importantly, however, although hydroxy-PP was identified in a screen for specific Cbr1 inhibitors, it was also found to inhibit Akr1C3, another enzyme capable
of Dox reduction *in vitro* (Kassner et al., 2008). While the inhibition constant of Dox-reduction in hepatic cytosol more closely matched Cbr1, the non-specificity of hydroxy-PP makes it difficult to conclusively attribute hepatic Doxol formation to Cbr1.

Genetic studies also suggest Cbr1 plays an important role in Doxol formation. While a homozygous-null deletion of the *cbr1* gene in mice is lethal, heterozygous mice with only a single copy of *cbr1* are viable and have reduced levels of Cbr1 mRNA and protein. Intriguingly, heterozygous mice showed less Dox-induced cardiotoxicity than wild type mice (Olson et al., 2003). Unfortunately, the effect of heterozygosity on the rate of Doxol formation was not assessed. Inversely, in a study of transgenic mice that overexpress Cbr1 in cardiac tissue, Cbr1 overexpression was correlated with both an increase in Doxol formation and cardiotoxicity (Forrest et al., 2000). Additionally, cancer cells expressing high concentrations of carbonyl reductase have been reported to be more resistant to the anti-proliferative effects of Dox, and small hairpin RNA knockdown of Cbr1 in breast cancer cells increased their sensitivity to Dox (Bains et al., 2013; Jo et al., 2017). These studies are consistent with Cbr1 playing a dominant role in Doxol formation *in vivo*, but genetic studies alone have significant limitations. Genetic interventions often have secondary effects, and any number of genes besides the original target might have their expression increased or decreased. This makes it difficult to deconvolute whether the observed changes in Dox metabolism are caused by the original intervention or by secondary effects on gene expression.
1.3: Carbonyl Reductase 3

Cbr3 is a syntenic homolog of Cbr1. The gene for Cbr3 was discovered more recently than Cbr1, and its enzymatic capabilities and physiological role are less well understood (Watanabe et al., 1998). Despite sharing 71% sequence identity, Cbr3 has a narrower range of substrates than Cbr1, although there is some overlap (Pilka et al., 2009). Cbr3 is known to be regulated by nuclear factor erythroid 2–related factor 2 (Nrf2), indicating it may play a role in maintaining cellular redox homeostasis and antioxidant stress response (Cheng et al., 2012; Ebert et al., 2010; Hu et al., 2006). Consistent with this, two mouse models with damage to different antioxidant systems exhibit over-expression of Cbr3 mRNA and protein. In one model, knock-out mice missing the gclm gene encoding the modifier subunit of glutamate-cysteine ligase (which catalyzes the rate limiting step in glutathione synthesis) express roughly 10-fold higher levels of Cbr3 than wild type mice (Haque et al., 2010).

Cbr3 was initially implicated in Dox reduction when genome wide analyses showed that certain allelic variants of Cbr3 were correlated with a higher rate of Dox-induced congestive heart failure and altered production of Doxol (Blanco et al., 2008; Fan et al., 2008). The in vitro effect of these variants on Doxol formation was studied and supported the conclusions of the population studies (Bains et al., 2010; Blanco et al., 2008). Subsequent studies using the Cbr3-overexpressing, gclm-null mice model further strengthened the link between Cbr3 and Doxol formation (Schaupp et al., 2015). Liver cytosol and isolated hepatocytes derived from gclm-null mice show elevated formation of Doxol, and gclm-null hepatocytes incubated with Dox produce a substance that is toxic to cultured myocytes.
1.4: Aldo-keto Reductase

The aldo-keto reductase (AKR) superfamily of proteins consists of monomeric, NADPH-dependent oxidoreductases that catalyze the reduction of aldehydes and ketones to their respective primary or secondary alcohols (Jin and Penning, 2007). There are 15 known human AKRs, and a number of them have been identified as potential Dox reductases (Jin and Penning, 2007; Nishinaka et al., 2003; Weber et al., 2015). Akr1A1 (aldehyde reductase), Akr1B1 (aldose reductase), Akr1B10, Akr1C3 (17β-hydroxysteroid dehydrogenase type 5), and Akr1C4 have all been demonstrated to catalyze the reduction of Dox to Doxol in vitro (Jin and Penning, 2007; Kassner et al., 2008). Of these, Akr1C3 catalyzes Dox reduction most efficiently, however, the Doxol-forming activity of Akr1C3 is 10-fold more sensitive to the inhibitor hydroxy-PP than the activity detected in cytosol (Kassner et al., 2008; Tanaka et al., 2005), reducing the likelihood that Akr1C3 plays a physiological role in Doxol formation. Pharmacological inhibition of AKRs in general and Akr1B1 and Akr1B10 specifically have successfully reduced Dox-associated cardiotoxicity and decreased the formation of Doxol; however these studies share the same vulnerabilities as those using hydroxy-PP to investigate Cbr1 (Behnia and Boroujerdi, 1999; Morikawa et al., 2015; Sonowal et al., 2017). The inhibitors used are unlikely to be truly specific to their AKR targets and are potentially inhibiting other oxidoreductases. Our study did not investigate the role of AKRs in Doxol formation.
1.5: Thioredoxin Reductase 1

In addition to Cbrs 1 and 3, our study investigated the role of thioredoxin reductase 1 (Tr1) in Doxol formation. Tr1 is a homodimeric, NADPH-dependent seleno-flavoprotein named for its unique ability to reduce the small redox protein thioredoxin (Arner and Holmgren, 2000). Besides the eponymous thioredoxin, Tr1 is capable of reducing a number of additional substrates, including the small molecules lipoic acid (Arner et al., 1996), lipid hydroperoxides (Bjornstedt et al., 1995), dehydroascorbate (May et al., 1997), ascorbyl free radical (May et al., 1998), alloxan, and menadione (Luthman and Holmgren, 1982). Tr1 and the thioredoxin system as a whole have been linked to Dox metabolism in the context of redox cycling of Dox (Ravi and Das, 2004; Ravi et al., 2005), but the effect of Tr1 on Doxol formation has never been studied. Additionally, liver-specific deletion of the mouse txnrd1 gene encoding Tr1 causes massive overexpression of Cbr3 mRNA and protein, potentially as a compensatory response to a lack of Tr1 (Bondareva et al., 2007; Suvorova et al., 2009). Due to its broad substrate specificity, as well as a potential substrate overlap with Cbr3, we evaluated Tr1 as another candidate for playing a role in Doxol formation.

1.6: Experimental Approach

Our work assessed the contributions of Cbr1, Cbr3, and Tr1 to Doxol formation in mouse liver cytosol. Initially, purified recombinant forms of these enzymes were tested for their ability to catalyze Dox-dependent NADPH oxidation and NADPH-dependent Doxol formation in vitro. Although all three enzymes
catalyzed Dox-dependent NADPH oxidation, only Cbr1 and Cbr3 catalyzed Doxol formation. Instead of Doxol formation, Tr1 catalyzed Dox-dependent generation of H₂O₂, probably via redox cycling. To directly test for a physiological role of the three enzymes in Dox metabolism, antibodies to the three proteins were developed and used to immunoclear the respective proteins from liver cytosol. Immunoclearing Cbr3 or Tr1 had no effect on Doxol formation by liver cytosol, suggesting these enzymes played no physiological role in Doxol formation. In contrast, immunoclearing Cbr1 eliminated 25% of the Doxol-forming activity in liver cytosol, providing the strongest evidence to date of a role for Cbr1 in Doxol formation in vivo. Importantly, however, the results showed that most of the Doxol-forming activity in liver cytosol was not Cbr1. Although pharmacogenetic evidence has suggested aldo-keto reductases may play a role (Morikawa et al., 2015; Sonowal et al., 2017), unequivocal identification of the remaining Doxol-forming activity is an important next step in developing strategies to reduce cardiotoxicity during Dox chemotherapy.
CHAPTER 2: Materials and Methods

2.1: Mouse liver fractionation

Mice were handled using procedures approved by Oregon State University IACUC. Adult mice were fasted overnight and euthanized by cervical dislocation. Livers were perfused in situ with PBS (150 mM NaCl, 5 mM NaPO₄ pH 7.4) to reduce blood content, removed to a petri dish, minced, and homogenized (Elvehjem, 5 strokes) in 10 volumes cold MOPS isolation buffer (200 mM sucrose, 10 mM Tris/MOPS pH 7.4, 1 mM EGTA) (Frezza et al., 2007). Homogenates were centrifuged for 10 min at 12,000g to remove nuclei and undisrupted cells. Clarified lysates were centrifuged for 30 min at 45,000g to remove mitochondria. The 45,000g supernatants were centrifuged for 1h at 100,000g to obtain microsomes (100,000g pellet) and cytosols (100,000g supernatant). Microsomes were resuspended in MOPS isolation buffer. Protein concentrations were determined by the Bradford method using bovine serum albumen as standard.

Ammonium sulfate fractionation was initiated by adding solid (NH₄)₂SO₄ to cytosol prepared from 13 pooled mouse livers to achieve a salt solution at 40% of saturation (1.64 M). After stirring 30 min at 4°C, the mixture was centrifuged for 30 min at 12,000g to obtain a 40% pellet and 40% supernatant. Solid (NH₄)₂SO₄ was added to the 40% supernatant and the above steps were repeated to achieve first a 50% pellet and supernatant, and subsequently a 60% pellet and supernatant. Precipitates were resuspended in 150 mM KCl, 50 mM KPO₄ pH 7.4. Fractions were stored at -20°C until assay.
2.2: Immunological procedures

Antisera were generated in New Zealand White rabbits, using full-length mouse Cbr1, Cbr3, and Tr1 with N-terminal 6XHis tags as antigen. Proteins were produced in BL21 bacteria using the expression vector pET28a and purified by Talon resin chromatography (Clontech, Mountain View, CA). Cbr3 and Tr1 immunizations and bleeds were done at the Oregon State University LARC. Cbr1 immunizations and bleeds were done by Pacific Immunology (Ramona, CA). Two rabbits were used for each protein. Antisera identification codes were assigned based on the source rabbit (Cbr1, 12081 and 12082; Cbr3, OB2 and OB4; Tr1, OB1 and OB3). Terminal bleeds (100-120 ml) were done by heart puncture. Raw antisera were prepared by allowing blood to coagulate at 4°C overnight, centrifuging the mixture at 5000g for 15 min, and storing 10-ml aliquots of supernatant at -70°C. Working aliquots were stored at 4°C, with 0.1% NaN₃ added as a preservative.

Raw Cbr3 antiserum cross-reacted with Cbr1 and raw Cbr1 antiserum cross-reacted with Cbr3 when used to probe immunoblots. To increase specificity, raw Cbr1 antiserum (12081) was cross-adsorbed to Cbr3-conjugated Affi-gel 15 (Bio-Rad, Hercules, CA) to obtain Cbr1-specific antiserum, and raw Cbr3 antiserum (OB4) was cross-adsorbed to Cbr1-conjugated Affi-gel to obtain Cbr3-specific antiserum (Fig. 4). Comparison of immunoblots probed with raw Cbr3 antiserum, Cbr3-specific antiserum, and Cbr1-specific antiserum showed that Cbr1 and Cbr3 migrated as a triplet during SDS-PAGE analysis of mouse liver cytosol, with the slower two bands representing Cbr1 and the faster band representing Cbr3 (see Fig. 9, Fig. 10, Fig. 12, and Fig. 13 for examples).
Figure 4. Preparation of affinity-purified Cbr3-specific antibody

(A) Purification scheme. Raw Cbr3 antiserum was passed over Cbr1-conjugated agarose beads to remove antibodies that cross-react with Cbr1 and obtain Cbr3-specific antiserum in the flow-thru. The latter was passed over Cbr3-conjugated agarose beads where bulk IgG were collected in the flow-thru and bound affinity-purified, Cbr3-specific antibody was eluted with acid. (B) Immunoblots of purified Cbr1 and Cbr3 protein probed with raw antiserum, Cbr3-specific antiserum, and affinity-purified, Cbr3-specific antibody. A similar scheme was used to prepare affinity-purified, Cbr1-specific antibody from raw Cbr1 antiserum.
To remove bulk immunoglobulins that would compete for Protein A binding during immunoclearing, Cbr1- and Cbr3-specific antisera were affinity-purified (Fig. 4). Briefly, 10 ml of specific antisera were incubated with 5 ml of Cbr1-conjugated or Cbr3-conjugated Affi-gel 10 beads (prepared as described below). After an overnight incubation at 4°C with rocking, the beads were rinsed several times with PBS, and bound antibodies were eluted with 150 mM NaCl, 20 mM HCl into tubes containing 100 µl of neutralizing 1 M KPO₄ pH 7.5 per 1.5 ml of eluate and stored at -20°C.

Immunoclearing of Cbr1 or Cbr3 was done using a non-covalent bridge of affinity-purified, Cbr1- or Cbr3-specific antibody bound to Protein A magnetic beads (Bio-Rad). Magnetic bead slurry (300 µl) was mixed with 1.5 ml affinity-purified Cbr1- or Cbr3-specific antibody and incubated overnight at 4°C with shaking, effectively saturating all available protein A on the beads with antibody. The excess antibody solution was removed, and the beads were washed several times with chilled PBS immediately before use. Beads pre-incubated only with PBS were used as mock-cleared controls. To immunoclear Cbr1 or Cbr3, 50 µl of antibody-coated beads was mixed with 120 µg of cytosolic liver protein in a final reaction volume of 100 µl 50 mM KPO₄ pH 7.5. Equivalent amounts of cytosolic protein were mixed with 50 µl PBS-washed beads as mock-cleared controls. After the mixtures were incubated overnight at 4°C with shaking, the beads were held against the tube walls magnetically, while the immunocleared and mock-cleared solutions were removed for immunoblot and enzymatic analyses.

Immunoclearing of Tr1 was done using affinity-purified Tr1 antibody that was covalently coupled to Affi-gel 15 (prepared as described below). Covalent coupling
was necessary because the molecular weights of Tr1 and IgG heavy chain were similar (50 kDa) and unavoidable leaching of some IgG using the non-covalent Protein A bridge method would complicate interpretation of immunoblots designed to measure the efficiency of Tr1 removal. Ethanolamine-coupled Affi-gel was used as mock-cleared controls. To immunoclear Tr1, 50 µl of Tr1 antibody-coupled Affi-gel was mixed with 300 µg of cytosolic liver protein in a final reaction volume of 100 µl homogenization buffer containing 10 mM HEPES, pH 7.4, 0.15 M KCl, 1 mM sodium-EDTA, 1 mM DTT, 0.2 mM Pefablock SC (Roche Diagnostics, Mannheim, Germany) (Kassner et al., 2008). Equivalent amounts of cytosolic protein were mixed with 50 µl ethanolamine-coupled beads as mock-cleared controls. After incubating the mixtures overnight at 4°C with shaking, beads were sedimented by centrifugation for 1 min at 2000 rpm in a table-top centrifuge, and the immunocleared and mock-cleared supernatants were removed for immunoblot and enzymatic analyses.

After the efficiency of immunoclearing was determined by immunoblotting, 12 µg-equivalents of immunocleared or mock-cleared cytosolic protein was assayed for Dox-dependent NADPH oxidation activity and NADPH-dependent Doxol forming activity as described below.

For immunoblot analyses, proteins were separated by SDS-PAGE and electroblotted to nitrocellulose membrane (Bio-Rad). Membranes were blocked overnight at 4°C in TBST (20 mM Tris pH 7.4, 150 mM NaCl, 0.1% Tween) containing 5% non-fat dried milk (NFDM). Primary antibody incubations were for at least 2 h at 25°C, using a 1:1000 dilution of raw antiserum or 1:300 dilution of affinity-purified antibody in TBST 5% NFDM. After four 5-min washes with TBST
5% NFDM, secondary antibody incubations were for at least 1 h at 25°C, using a 1:5000 dilution of goat anti-rabbit HRP-conjugated IgG (Bio-Rad). Following four TBST washes, blots were developed with Western Lightning enhanced chemiluminescent substrate (Santa Cruz Biotechnology, Dallas, TX) and imaged using a ChemiGenius Bio-imaging system (Syngene, Frederick, MD). Protein band intensities were determined using Syngene Genetools software.

Conjugation of purified enzymes and antibodies to agarose was done using Affi-gel 15 beads (Bio-Rad). Prior to coupling, proteins and antibody were exchanged into 50 mM KPO₄ pH 8.0, 150 mM KCl, using an Econo-Pac 10DG desalting column (Bio-Rad). Cold Affi-gel 15 slurry (10 ml) from the source vial was transferred to a glass-fritted funnel on a vacuum flask, and rapidly washed twice with 10 ml cold dH₂O, and once with 10 ml cold 50 mM KPO₄ pH 8.0, 150 mM KCl. The washed beads were immediately resuspended in 5 ml purified enzyme or antibody and agitated gently in the funnel for 1 h at room temperature to allow coupling. For a 5-ml coupling reaction, 10-15 mg of purified protein was used. After the coupling reaction, beads were washed with 10 ml water, resuspended in 10 ml 1 M ethanolamine, and gently agitated for 30 min at room temperature to block any remaining amine-reactive sites of the beads. The beads were rinsed twice and stored as a 50% slurry in 50 mM KPO₄ pH 8.0, 150 mM KCl at 4°C.

2.3: Enzyme assays

Dox-dependent NADPH oxidation was measured in UV-transparent 96-well plates by tracking A₃₄₀ using a Biotek Synergy 2 Multi-Mode Microplate Reader.
(Winooski, VT). Reaction cocktails contained 50 mM KPO₄ pH 7, 0-200 µM Dox, 1-2 µM recombinant enzyme or 50-100 µg of liver protein, and 200 µM NADPH (added last) in a total volume of 150 µl. Absorbance was monitored for 1 h at 37°C, and the molar amount of NADPH oxidized was calculated using an extinction coefficient of 6220 M⁻¹cm⁻¹ and estimated pathlength of 3 mm. The rate of NADPH oxidation in the absence of Dox was subtracted from rates measured in the presence of Dox.

NADPH-dependent Doxol formation was measured by LC-MS/MS. Reaction cocktails were prepared as described above. After a 1-h incubation at 37°C, reactions were stopped and proteins precipitated by adding chilled sulfosalicylic acid to 0.5%. Precipitated protein was removed by centrifugation for 10 min at 12,000g. Supernatants were analyzed using a SCIEX 3200 Q TRAP LC-MS/MS system equipped with Shimadzu LC-20AD pumps, SIL-20A model autosampler, CTO-10ASvp model column oven, and CBM-20A model system controller. Dox was separated from Doxol using an Agilent Poroshell 120 PFP reversed-phase column (Santa Clara, CA), 10 µl sample injection volume, 0.3 ml/min flow rate, and 10-90% acetonitrile gradient applied over 10 minutes. For Dox, the Q1 parent peak was set to 544.4 Da and Q3 peaks were set to 397.3, 379.3, and 130.1 Da. For Doxol, Q1 was set to 546.4 Da and Q3 peaks were set to 399.3, 381.3, and 130.1 Da.

Kinetic parameters were independently calculated four ways: non-linear least squares regression, Lineweaver-Burk, Eadie-Hoffstee, and Hanes-Woolf analysis. For mouse liver cytosol, Vₘₐₓ was calculated instead of Kₗₐₜ. Raw data is provided in Appendix A and B.
H$_2$O$_2$ formation was measured using an Amplex red-based assay kit (Invitrogen, Carlsbad, CA). Purified Cbr1 and Tr1 (1 µM) were mixed with 50 µM Dox in 50 mM KPO$_4$ pH 7.4. NADPH (200 µM) was added to initiate the reaction and bring the final volume to 100 µl. Samples were combined with 100 µl of Amplex red working solution (100 µM Amplex red, 200 U/ml HRP, 50 mM KPO$_4$ pH 7.4). Using a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Waltham, MA), samples were excited at 470 nm, and emission in the far-red range (665-720 nm) was tracked. A standard curve of 0-1 mM H$_2$O$_2$ was used to quantify H$_2$O$_2$ production by samples. The same procedure was followed to measure H$_2$O$_2$ production by 30 µg of liver cytosolic or microsomal protein.
CHAPTER 3: Results

3.1: Dox-dependent NADPH oxidation and NADPH-dependent Doxol formation by purified Cbr1, Cbr3 and Tr1

Previous research (Kassner et al., 2008; Schaupp et al., 2015) suggested that the enzymes Cbr1, Cbr3, and Tr1 were promising candidates for catalyzing reduction of Dox to Doxol (Fig. 3). As an initial assessment of these candidate enzymes, recombinant mouse Cbr1, Cbr3, and Tr1 were purified and tested for their ability to catalyze Dox-dependent NADPH oxidation. All three enzymes were active (Fig. 5A, Fig. 5B). After initial confirmation of enzyme activity, kinetic parameters for Dox-dependent NADPH oxidation by these enzymes were determined and are summarized in Table 1. Cbr1 had a $K_{\text{cat}}$ 10-fold greater than Cbr3 and 1.5-fold greater than Tr1. However, due to its lower $K_m$, Tr1 had a 2.8-fold higher catalytic efficiency ($K_{\text{cat}}/K_m$) than Cbr1.

Although spectrophotometric analysis of NADPH oxidation was an expedient way of assessing whether an oxidoreductase used Dox as a substrate, it did not establish that the product of the reaction was Doxol. For example, ring keto groups on Dox could alternatively serve as electron acceptors. We thus used LC-MS/MS analysis to directly measure the production of Doxol by Cbr1, Cbr3, and Tr1. Pilot analyses with standards established that Doxol could be distinguished from its precursor and that the Doxol M/Z peaks were proportional to the amount of Doxol added to the mixture (Fig. 6). Using the LC-MS/MS assay, the amount of Doxol formed by each enzyme during a 1-h incubation with 200 µM NADPH and Dox was determined (Fig. 7). Both Cbr1 and Cbr3 produced Doxol. In contrast, despite showing a high rate of Dox-dependent NADPH oxidation, Tr1 produced no Doxol
Figure 5: Dox-dependent NADPH oxidation by purified recombinant Cbr1, Cbr3, and Tr1

(A) Time-course of NADPH oxidation by Cbr1, Cbr3 and Tr1 in the presence (dashed line) or absence (solid line) of Dox. Reaction conditions were 1 µM Cbr1, 1.9 µM Cbr3 or 1.9 µM Tr1; 0 µM or 200 µM Dox; and 200 µM NADPH. A$_{340}$ was monitored for 1 h at 37°C. (B) Rate of Dox-dependent NADPH oxidation by purified Cbr1, Cbr3, and Tr1. The rates shown were calculated from the linear phase of each time course, and were corrected by subtracting the rates observed in the absence of Dox. Error bars represent one standard deviation and n=3 for all reactions.
Table 1: Kinetic parameters Km, $k_{cat}$, and $k_{cat}/Km$ for Dox-dependent NADPH oxidation by purified Cbr1, Cbr3, and Tr1.

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<tr>
<th>Enzyme</th>
<th>Analysis Method</th>
<th>$R^2$</th>
<th>Km (µM)</th>
<th>$k_{cat}$ (s$^{-1}$) $\times 10^3$</th>
<th>$k_{cat}/Km$ (µM$^{-1}$s$^{-1}$) $\times 10^6$</th>
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$^1$The average of the four analysis methods is shown in grey.

$^2$Not included in average due to poor correlation co-efficient ($R^2$).
Figure 6: Representative LC-MS/MS chromatograms of Dox and Doxol reference standards
Standards contained 200 µM Dox and increasing concentrations of Doxol. Retention time was 5.3 min for Dox and 4.9 min for Doxol. The areas of both the tall parental ion peak (546.4 Da) and shorter secondary ion peaks (399.3, 381.3, and 130.1 Da) were used as standards to quantify Doxol levels in experimental samples.
Figure 7: NADPH-dependent Doxol formation by purified recombinant Cbr1, Cbr3, and Tr1

Reaction conditions were 1 µM enzyme, 200 µM Dox and 200 µM NADPH. Incubations were for 1 h at 37°C. Doxol levels were measured by LC-MS/MS. Error bars represent one standard deviation and n=3 for all reactions.
during the reaction. This result revealed that tracking Dox-dependent NADPH oxidation alone was not sufficient to draw conclusions regarding the formation of Doxol. Kinetic parameters for Doxol formation by Cbr1 and Cbr3 were determined and compared to kinetic parameters for Doxol formation by mouse liver cytosol (Table 2). Cbr1 and Cbr3 had similar Km values, but Cbr1 was about 400-fold more efficient at Doxol formation than Cbr3. The Km values for Doxol formation exhibited by purified Cbr1 and Cbr3 were similar to those exhibited by liver cytosol, consistent with these enzymes playing a role in vivo.

The initial validation of Doxol formation by Cbr1 and Cbr3 used a high concentration of Dox (200 µM) to ensure enough Doxol would be produced. In that experiment Cbr1 produced Doxol formation much more efficiently than Cbr3 (Fig. 7), however, the concentrations of Dox used were too high to be considered physiologically relevant as Dox concentration in the high nanomolar-low micromolar range (Speth et al., 1987). The rate of Doxol formation by Cbr1 and Cbr3 at lower concentrations of Dox was determined in the course of assessing the kinetics of Doxol formation by Cbr1 and Cbr3, and Cbr1 was again found to produce Doxol more efficiently than Cbr3. In the presence of 1 µM Dox and 200 µM NADPH Cbr1 produced Doxol at a rate of 1.9 µM Doxol/h and Cbr3 produced Doxol at a rate of .0034 µM Doxol/h. Although the amount of Doxol produced using 1 µM Dox was quantifiable when purified enzyme was used, 200 µM Dox was used when quantifying Doxol formation by cytosols (described below), to facilitate detection of product when Doxol-forming activity was low.
Table 2: Kinetic parameters $K_m$, $k_{cat}$, and $k_{cat}/K_m$ for NADPH-dependent Doxol formation by purified Cbr1 and Cbr3, as well as $K_m$ and $V_{max}$ for NADPH-dependent Doxol formation by mouse liver cytosol.

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<th>Enzyme</th>
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$^1$The average of the four analysis methods is shown in grey.

$^2$Lineweaver-Burk parameters for Cbr3 were outliers and not included in average.

$^3$Lineweaver-Burk analysis of cytosol data failed to converge.
3.2: Doxorubicin-dependent redox cycling catalyzed by Tr1

Since Dox-dependent NADPH oxidation by Tr1 was not associated with Doxol formation, we hypothesized that Tr1 was instead catalyzing NADPH oxidation by a redox cycling mechanism (Fig. 2). To test this hypothesis, Tr1 and Cbr1 were incubated with Dox and NADPH, and the amount of H$_2$O$_2$ produced over the course of the reaction was measured using Amplex Red reactivity as an indicator. The results (Fig. 8) showed that in the presence of Dox and NADPH, Tr1 generated significant amounts of H$_2$O$_2$. Cbr1 also produced H$_2$O$_2$, but at a much slower rate. The efficient formation of H$_2$O$_2$, along with the lack of Doxol formation, suggested Tr1 was indeed catalyzing Dox-dependent redox cycling. The thioredoxin system has previously been linked to the redox cycling of anthracyclines (Ravi and Das, 2004; Ravi et al., 2005). Our findings support this conclusion.

3.3: Subcellular localization of Dox-metabolizing activities in liver

To confirm the cytosolic localization of Cbr1, Cbr3 and Tr1, and to determine whether the enzymes catalyzing Dox-dependent NADPH oxidation and Doxol formation were similarly cytosolic, 45,000g liver supernatants were fractionated into their microsomal and cytosolic components by centrifugation at 100,000g. Equal amounts of protein from each component were analyzed for Cbr1, Cbr3 and Tr1 protein by immunoblotting, Dox-dependent NADPH oxidation by spectrophotometry, and Doxol-forming activity by LC-MS/MS. Immunoblot analyses (Fig. 9A) confirmed that all three proteins were primarily cytosolic. However, when Dox-dependent NADPH oxidation activity was assayed, most of the activity present in the
Figure 8: $\text{H}_2\text{O}_2$ formation by Cbr1 and Tr1

Reaction conditions were 1 µM Cbr1 or Tr1, 50 µM Dox and 200 µM NADPH; incubations were for 1 h at room temperature.
Figure 9: Subcellular localization of Cbr1, Cbr3 and Tr1 protein; Dox-dependent NADPH oxidation activity; NADPH-dependent Doxol-forming activity; and H₂O₂ formation in mouse liver

(A) Immunoblot analysis of Cbr1, Cbr3, and Tr1 protein in indicated supernatants (S) and pellets (P): 45,000g S (a mixture of cytosol and microsomes), 100,000g S (cytosol), and 100,000g P (microsomes). Lysates from gclm-null mice were used to facilitate Cbr3 localization. (B) Dox-dependent NADPH oxidation by indicated fractions of wild-type liver. Reaction conditions were 50 µg fraction protein, 200 µM NADPH and 200 µM Dox. Incubations were for 1 h at 37°C. (C) NADPH-dependent Doxol formation by indicated fractions of wild-type liver lysates. Reaction conditions and incubations were as described in B. Doxol levels were measured by LC-MS/MS. (D) H₂O₂ formation by indicated fractions of wild-type liver. Reaction conditions were 30 µg fraction protein, 200 µM NADPH and 50 µM Dox. Incubations were for 1 h at 25°C. Error bars represent one standard deviation; n=3 for all reactions; * indicates p<0.05 by Student’s t test.
original 45,000g supernatant was recovered in the microsomal fraction, and only 5% of the activity was recovered in the cytosolic fraction (Fig. 9B). In contrast, Doxol formation was almost entirely cytosolic (Fig. 9C).

We speculated that the high levels of Dox-dependent NADPH oxidation activity observed in microsomal fractions was due to redox cycling, as microsomes contain cytochrome p450 proteins which are known to carry out one-electron reductions of quinones to semiquinone radicals for other substrates known to undergo redox cycling (Hopkins, 2016). To test this hypothesis, we measured the production of H$_2$O$_2$ by cytosolic and microsomal fractions. As shown in Fig. 9D, microsomal fractions produced significant amounts of H$_2$O$_2$, while cytosolic fractions did not, suggesting that the Dox-dependent NADPH oxidation activity seen in the microsome was caused by redox cycling. Since Tr1 was primarily cytosolic, it likely was not a major contributor to total Dox-dependent redox cycling in the liver.

### 3.4: Quantification of Cbr1 and Cbr3 protein in wild type and gclm-null mouse liver cytosol

By analyzing dilutions of purified Cbr1 and Cbr3 in parallel with fixed amounts of cytosolic protein from wild type and gclm-null livers, we were able to quantify the relative levels of these two enzymes in liver cytosol (Fig. 10). In wild type liver cytosol, Cbr1 accounted for 0.1% of total cytosolic protein and was 300-fold more abundant than Cbr3. In gclm-null cytosol, Cbr1 accounted for 0.2% of cytosolic protein and was 60-fold more abundant than Cbr3. In gclm-null cytosols, the levels of both proteins were increased. Cbr3 increased 10-fold, while Cbr1 doubled. The 10-fold increase in Cbr3 protein matched the increase in Cbr3 protein reported by
Fig. 10. Relative levels of Cbr1 and Cbr3 in wild type and gclm-null mouse liver cytosol

Wild type mouse liver cytosolic protein (52.5 µg) and gclm-null mouse liver cytosolic protein (75 µg) and known quantities of purified recombinant Cbr1 and Cbr3 were analyzed in parallel by SDS-PAGE and immunoblotting. Blots were probed with raw Cbr3 antiserum, which recognizes both Cbr3 (fastest band, solid arrow) and Cbr1 (slower-migrating bands, open arrow). Densitometry was used to quantify Cbr1 and Cbr3 band intensities in each lane (number shown at bottom).
Haque et al. (2010). It should be noted that only one wild type and one gclm-null cytosol were analyzed, so these results were not statistically evaluated and were only used as a rough guide for assessing protein levels when immunoclearing.

3.5: Effect of immunoclearing Cbr1 on Doxol-forming activity in mouse liver cytosol

As demonstrated above, purified Cbr1 and Cbr3 were able to catalyze NADPH-dependent Doxol formation, with Cbr1 catalyzing the reaction much more efficiently than Cbr3. However, these in vitro analyses did not establish the extent to which these enzymes contributed to Doxol formation in the complicated milieu of the cytosol. Post-translational modifications, accessory proteins, and small molecule effectors could impact the formation of Doxol by these enzymes in vivo. Additionally, other Doxol-forming enzymes might exist in the cytosol, diluting the contributions of Cbr1 and Cbr3 to overall activity. As discussed previously, genetic interventions, such as gene knock-outs and siRNA, are often used to study the role of specific proteins within an organism or cell; however, such interventions often have unpredictable effects on the transcriptome. For example, knocking out the txnrd1 gene encoding Tr1 in mouse liver results in significant overexpression of many mRNAs, including a 50-fold increase in Cbr3 mRNA and smaller increase in Cbr1 mRNA (Bondareva et al., 2007; Suvorova et al., 2009). A similar phenomenon occurs in gclm-null mice, where both Cbr3 and, to a lesser extent, Cbr1 mRNAs are upregulated (Haque et al., 2010).

As an alternative to genetic interventions, we developed an immunoclearing approach to analyze the roles of Cbr1, Cbr3 and Tr1 in Doxol formation. This
approach avoided complications that could arise from altered gene expression in knock-out models, while still requiring the enzymes to function within the context of the cytosol.

Protein A magnetic beads preincubated with Cbr1-specific antibody were used to immunoclear Cbr1 from six mouse liver cytosols. Beads preincubated only with buffer served as mock-cleared controls. The amounts of Cbr1 present in Cbr1-cleared and mock-cleared cytosols were determined by immunoblot analysis (Fig. 11A). Densitometry showed that on average 67% of Cbr1 protein was removed during immunoclearing. To confirm the specificity of the immunoclearing reaction, immunoblots were re-probed with antibody against the small redox protein thioredoxin (Trx1). As expected, there was no significant difference in the amount of Trx1 present in Cbr1-cleared and mock-cleared cytosols (Fig. 11A).

Equivalent amounts of Cbr1-cleared and mock-cleared cytosolic protein were incubated with NADPH and Dox, and the amount of Doxol produced during a 1 h reaction was measured by LC-MS/MS (Fig. 11B). Five of six cytosols showed a statistically significant reduction in the amount of Doxol produced by Cbr1-cleared cytosol, compared to mock-cleared cytosol. When averaged, Doxol formation by the Cbr1-cleared cytosols was 21% lower than the mock-cleared group. Since the immunoblotting analyses showed that the immunoclearing procedure removed only 67% of the Cbr1 in the cytosol, we calculated that 31% of the Doxol-forming activity (21%/0.67) in mouse liver cytosol was attributable to Cbr1. When the immunoclearing analyses were repeated using the same cytosols and independently prepared new cytosols, slightly different estimates for the amount of activity
Figure 11: Immunoclearing of Cbr1 and its effect on Doxol-forming activity in mouse liver cytosol

(A) Immunoblot analyses of Cbr1 in mock-cleared and Cbr1-cleared cytosols from six wild-type mice. Cbr1 protein levels were determined by densitometry, and the percent of Cbr1 protein remaining after immunoclearing, relative to mock-cleared samples, is shown at bottom. Thioredoxin (Trx1) levels were determined in parallel as a control. (B) NADPH-dependent Doxol-forming activity in mock-cleared and Cbr1-cleared cytosols. Reaction conditions were 12 µg-equivalent of cytosolic protein, 200 µM Dox and 200 µM NADPH. Incubations were for 1 h at 37°C. Doxol levels were measured by LC-MS/MS. The percent of Doxol-forming activity remaining after immunoclearing, relative to mock-cleared samples, is shown at bottom. Error bars represent one standard deviation; n=3 for all reactions; * indicates p<0.05 by Student’s t test.
attributable to Cbr1 were obtained, ranging from 20% to 31%. By averaging the results from all experiments, we calculated that 23.4% ± 13.7% of Doxol-forming activity (roughly 25%) was attributable to Cbr1. The immunoclearing results thus provided unequivocal evidence that Cbr1 contributed significantly to Doxol formation in liver. Importantly, however, even with the majority of Cbr1 cleared from the cytosol, significant amounts of the Doxol-forming activity remained.

3.6: Effect of immunoclearing Cbr3 and Tr1 on Dox-dependent NADPH oxidation and Doxol-forming activity in mouse liver cytosol

The above results showed that Cbr1 accounted for about 25% of the total Doxol-forming activity in liver cytosol. To investigate whether Cbr3 and Tr1 contributed to the remaining unaccounted-for Doxol-forming activity, these proteins were also selectively immunocleared from mouse liver cytosol. Immunoblot analysis of the levels of Cbr3 and Tr1 in mock-cleared and immunocleared cytosols showed near-total clearing of Cbr3 and Tr1 was achieved (Fig. 12A).

Equal amounts of mock-cleared and immunocleared cytosolic protein were assayed for Dox-dependent NADPH oxidation activity and Doxol-forming activity. No significant difference in the rate of Dox-dependent NADPH oxidation was observed in Cbr3- or Tr1-cleared cytosols, relative to mock-cleared cytosols (Fig. 12B). Thus, although purified Tr1 efficiently catalyzed Dox-dependent NADPH oxidation in vitro, Tr1 did not contribute to Dox-dependent NADPH oxidation in the context of the cytosol. Clearing Cbr3 and Tr1 also had no effect on cytosolic Doxol-forming activity (Fig. 12C). Thus, neither Cbr3 nor Tr1 played a discernible role in Dox metabolism in liver cytosol.
**Figure 12: Immunoclearing Cbr3 and Tr1 from mouse liver cytosols and effect on Dox-dependent NADPH oxidation and NADPH-dependent Doxol formation**

(A) Immunoblot analysis of Cbr3 and Tr1 in mock-cleared and immunocleared mouse liver cytosols. The Cbr3 immunoblots were probed with either raw anti-Cbr3 antiserum (top) or Cbr3-specific antiserum (bottom). (B) Dox-dependent NADPH oxidation by mock-cleared and immunocleared cytosols. Reaction conditions were 45 µg-equivalent of cytosolic protein, 200 µM Dox, 200 µM NADPH, incubations were for 1 h at 37°C. Error bars represent one standard deviation; n=3 for all reactions. (C) NADPH-dependent Doxol formation by mock-cleared and Cbr3- or Tr1-cleared cytosols. Reaction conditions and incubations were as described in B. Doxol levels were measured by LC-MS/MS. Triplicate incubations for each cytosol were pooled and analyzed as one sample.
3.7: Doxol formation in txnrd1-null mouse liver cytosol

Cbr3 was implicated as a potential Dox reductase when it was observed that gclm-null mice, which have elevated levels of Cbr3 protein, have higher rates of Doxol formation (Schaupp et al., 2015). Interestingly, liver-specific deletion of the txnrd1 gene also resulted in greatly elevated levels of Cbr3 and mildly elevated levels of Cbr1 (Fig. 13A). We investigated whether Cbr3 contributed significantly to Doxol formation in txnrd1-null tissues that overexpressed Cbr3. Consistent with Cbr3 playing a role in Doxol formation, we found that txnrd1-null cytosols produced significantly more Doxol, but the effect was small (35% increase) relative to the large increase in Cbr3 protein (Fig. 13B).

To ascertain whether the increased Doxol-forming activity observed in txnrd1-null cytosols was attributable to Cbr3, Cbr1, or both enzymes, we immunocleared Cbr3 and Cbr1 individually and together from four txnrd1-null cytosols (Fig. 14A). Total clearing of Cbr3 was achieved in all cytosols, while clearing of Cbr1 was less thorough (about 55%) and more variable. Clearing Cbr3 had no effect on Doxol formation, while clearing Cbr1 again led to a reduction in Doxol formation. Clearing Cbr1 and Cbr3 simultaneously did not lead to any difference in Doxol formation when compared to clearing Cbr1 alone (Fig. 14B). This suggests that even when Cbr3 was highly overexpressed, it was not responsible for any of the observed Doxol-forming activity in liver cytosol. The increased Doxol formation observed in txnrd1-null livers was thus likely due to the increased expression of Cbr1 or some as yet unidentified enzyme.
Figure 13: Cbr1 and Cbr3 levels and Doxol-forming ability of wt and txnrld-null mouse liver cytosols

(A) Immunoblot analysis of Cbr3 and Cbr1 in wild-type and txnrld-null mouse liver cytosols; 18 µg of total cytosolic protein loaded per lane. The top panel shows a blot probed with Cbr3-specific antibody. The bottom panel shows the same blot re-probed with Cbr1-specific antibody; because the blot was not stripped, the fast-migrating Cbr3 signal remained visible. (B) NADPH-dependent Doxol formation by wild-type and txnrld-null cytosols. Reaction conditions were 12 µg-equivalent of cytosolic protein, 200 µM Dox and 200 µM NADPH; incubations were for 1 h at 37°C. Doxol levels were measured by LC-MS. Error bars represent one standard deviation; n=4 for all reactions; * indicates p<0.05 by Student’s t test.
Figure 14: Immunoclearing of Cbr1 and/or Cbr3, and its effect on Doxol-forming activity in txnrd1-null mouse liver cytosols that overexpress Cbr3

(A) Immunoblot analysis of immunocleared txnrd1-null cytosols. Cbr1 and Cbr3 protein levels were determined by densitometry, and the % protein remaining after immunoclearing, relative to mock-cleared samples, is shown at bottom. (B) NADPH-dependent Doxol formation by mock-cleared, Cbr3-cleared, Cbr1-cleared, and Cbr3- and Cbr1-cleared txnrd1-null cytosols. Reaction conditions and incubations were as described in B, except 50 µM Dox was used. Doxol levels were measured by LC-MS/MS. The % Doxol-forming activity remaining after immunoclearing, relative to mock-cleared samples, is shown at bottom. Error bars represent one standard deviation; n=4 for all reactions; * indicates p<0.05 by Student’s t test.
3.8: Ammonium sulfate fractionation of Doxol-forming activity in liver cytosol

Having shown that only 25% of the Doxol-forming activity in liver cytosol was attributable to Cbr1, most of the total Doxol-forming activity remained unidentified. As an initial means of characterizing this remaining activity, we fractionated liver cytosol by ammonium sulfate precipitation. SDS-PAGE analysis revealed that each fraction had a distinct protein profile (Fig. 15A). Immunoblot analysis revealed that Cbr1 was found primarily in the 60% supernatant (Fig. 15B), whereas Doxol-forming activity was found primarily in the 60% pellet and 50% pellet (Fig. 15C). The finding that peak Doxol-forming activity was found in an ammonium sulfate fraction containing relatively little Cbr1 independently corroborated the conclusion of the immunoclearing experiment that enzymes other than Cbr1 comprised the majority of the Doxol-forming activity in liver cytosol.
Figure 15: Cbr1 levels and Doxol-forming activity in ammonium sulfate-fractionated mouse liver cytosol

(A) Coomassie-stained SDS-PAGE analysis of proteins in ammonium sulfate fractions; 10 µg of protein loaded per lane. (B) Immunoblot analysis of Cbr1 in ammonium sulfate fractions; 10 µg of protein loaded per lane. (C) Relative amount of Doxol produced by each fraction. Reaction conditions were 12 µg fraction protein, 200 µM Dox and 200 µM NADPH; incubations were for 1 h at 37°C. Doxol levels were determined by LC-MS/MS. Error bars represent one standard deviation; n=3 for all reactions; na, not assayed.
CHAPTER 4: Discussion

When Cbr3 or Tr1 protein was immunocleared from liver cytosol, no reduction in Doxol-forming activity was observed, indicating that these enzymes did not contribute to Doxol formation by liver cytosol. In addition, immunoclearing these enzymes also had no effect on Dox-dependent NADPH oxidation. Overall, these results suggested that Cbr3 and Tr1 play no discernible role in the hepatic metabolism of Dox. When Cbr1 was immunocleared from liver cytosol, 25% of the Doxol-forming activity was removed, indicating that 25% of the Doxol-forming activity was attributable to the Cbr1 polypeptide. Importantly, 75% of the Doxol-forming activity in liver cytosol remained after Cbr1 was removed by immunoclearing, indicating that other enzymes constituted the majority of the Doxol-forming activity. This conclusion was further supported by ammonium sulfate fractionation results that showed that peak levels of Doxol-forming activity were detected in fractions that contained little Cbr1.

Identification of the enzyme or enzymes that constitute this remaining activity is important, as any enzyme that forms Doxol represents a potential pharmacological target for reducing Dox-associated cardiotoxicity. Another enzyme capable of efficiently catalyzing Doxol formation in vitro is aldo-keto reductase 1C3 (Akr1C3) (Kassner et al., 2008). However, the Doxol-forming activity of Akr1C3 is 10-fold more sensitive to the inhibitor hydroxy-PP than the activity detected in cytosol (Kassner et al., 2008; Tanaka et al., 2005), reducing the likelihood that Akr1C3 plays a physiological role in Doxol formation. Other aldo-keto reductases potentially play a role in Dox metabolism. For example, pharmacogenetic studies have suggested
Akr1B10 or Akr1B1 may mediate the cardiotoxic effects of Dox (Morikawa et al., 2015; Sonowal et al., 2017). However, as the drugs used in these studies may have targeted other oxidoreductases, it is unclear whether the described effects were mediated by Akr1B10 or Akr1B1. The immunoclearing approach described in this study would be one way to assess the contributions of various Akr proteins to Dox metabolism. However, identification of the enzyme(s) responsible for the unaccounted portion of hepatic Doxol formation likely will require exhaustive fractionation of cytosolic proteins to achieve purity, biochemical identification of specific proteins, and subsequent immunoclearing validation.

Cbr3 mRNA is the most highly induced message in both txnrd1-null (Bondareva et al., 2007; Suvorova et al., 2009) and gclm-null tissues (Schaupp et al., 2015). It was suggested that Cbr3 might play a role in Doxol formation based on the observations that cytosols from gclm-null livers and isolated hepatocytes had elevated levels of Cbr3 protein and elevated levels of Doxol-forming activity, that gclm-null hepatocytes incubated with Dox produced a substance toxic to myocytes and produced higher levels of Doxol, and that purified Cbr3 was able to reduce Dox to Doxol in vitro (Bains et al., 2010). Our current findings show that purified Cbr1 was 400-fold more efficient than Cbr3 in converting Dox to Doxol, and that immunoclearing Cbr1, but not Cbr3, from either wild type or txnrd1-null cytosols removed a significant fraction of the measurable Doxol-forming activity. In view of the current findings, we suspect that the elevated Doxol-forming activity in gclm-null livers was due to Cbr1, which is also induced in both gclm-null and txnrd1-null livers, although not to the same extent as Cbr3. Based on immunoblot analyses, Cbr1 was
much more abundant than Cbr3 in wild-type extracts and remained more abundant than Cbr3 even in gelm-null and txnrd1-null livers that overexpress Cbr3.

Olson et al. (2003) showed that mice systemically heterozygous for a cbr1 gene deletion mutation are more resistant to the cardiotoxic effects of Dox, suggesting that halving the amount of Cbr1 in the cytosol was cardioprotective. As our results indicated that only 25% of the total Doxol-forming activity in wild-type cytosol was attributable to Cbr1, heterozygosity for the cbr1-null mutation would be expected to result in only a 12.5% reduction in total Doxol-forming activity. Thus, more experiments are necessary to understand the cardioprotective effect of cbr1-null heterozygosity during Dox treatment.

Jo et al. (Jo et al., 2017) showed that co-administration of the Cbr1-inhibiting drug hydroxy-PP-Me during Dox treatment of rodents results in greater chemotherapeutic activity against implanted tumors and lower cardiotoxicity. They concluded that Cbr1 plays a major role in converting Dox to cardiotoxic Doxol. Our current results are consistent with a role for Cbr1 in Doxol formation, but it is difficult to explain how inhibition of Cbr1 alone results in reduced cardiotoxicity when Cbr1 constitutes only 25% of the total Doxol-forming activity in liver cytosol. It is possible that Cbr1 constitutes a much higher percentage of total Doxol-forming activity in heart or other tissues, but we consider it more likely that the hydroxy-PP-Me is not specific for Cbr1 and instead inhibits a broader spectrum of NADPH-dependent oxidoreductases, including the unidentified enzymes responsible for 75% of the Doxol-forming activity in liver cytosol. Nevertheless, the hydroxy-PP-Me study is extremely important because it suggests that development of drugs that
inhibit Doxol-forming enzymes represents a promising strategy for combating cardiotoxicity during Dox chemotherapy.

In addition to evaluating the roles of three candidate enzymes in Doxol formation, the current study revealed that Dox participated in NADPH-dependent redox cycling reactions that generate H$_2$O$_2$, that the redox cycling activity was most abundant in microsomes but also was present in cytosol, and that, although purified Tr1 efficiently catalyzed redox cycling in vitro, immunoclearing Tr1 from cytosol had no effect on the observed redox cycling activity. Tr1 was thus not a significant contributor to the Dox-dependent redox cycling activity observed in liver lysates. Identifying the Dox-dependent redox cycling activity is important because redox cycling may contribute to the chemotherapeutic and/or cardiotoxic activities of Dox. For example, Dox is known to intercalate into DNA and if it were to participate in redox cycling reactions from this location, it could create localized high concentrations of DNA-damaging reactive oxygen species and thereby arrest replication.
CHAPTER 5: Conclusion

The purpose of the study was to assess the role of Cbr1, Cbr3, and Tr1 in the conversion of Dox to Doxol by mouse liver cytosol using a novel immunoclearing approach. The three enzymes selected for study were all linked to Dox metabolism in some way, with Cbr1 in particular having been referred to previously as the “predominant doxorubicin reductase in the human liver” (Kassner et al., 2008). This study describes a novel technique wherein antibodies against target enzymes were derived and used to selectively immunoprecipitate the enzymes from cytosol. Comparing the Doxol-forming activity of the mock-cleared and immunocleared cytosols thus allowed us to draw conclusions about the relative contributions of the enzymes to Doxol formation. The advantage of the approach is its specificity, unlike previous studies which relied on pharmacological inhibition or genetic alterations.

Initial in vitro analyses revealed that purified recombinant Cbr1 and Cbr3 were capable of catalyzing Doxol formation. Both enzymes had $K_m$ values roughly consistent with the $K_m$ for Doxol formation by liver cytosol, but Cbr1 was approximately 400-fold more efficient than Cbr3. Purified Tr1 was capable of catalyzing Dox-dependent NADPH oxidation but did not produce any Doxol. This discrepancy likely was due to redox cycling, as Tr1 produced significant amounts of reactive oxygen species when incubated with NADPH and Dox.

Following characterization of their in vitro activity, we developed an immunoclearing approach to assess the contributions of Cbr1, Cbr3 and Tr1 to Doxol formation in vivo. The most important finding of this study was that Cbr1 was only responsible for 25% of the Doxol-forming activity in mouse liver cytosol. Previous
studies had seemingly settled on Cbr1 as the major source of Doxol formation, but our result showed that while Cbr1 plays a role, the majority of the Doxol-forming activity in liver cytosol remains unidentified. This is important, as any attempts to reduce Doxol formation by inhibiting Cbr1 may be less effective unless steps are taken to inhibit the remaining Dox-reductases as well.

The current study also conclusively demonstrated that neither Cbr3 nor Tr1 played a significant role in Dox metabolism. This contradicted earlier studies which linked Cbr3 to Doxol formation.

The current study also showed that recombinant Tr1 was capable of catalyzing Dox-dependent redox cycling. The immunoclearing results, however, suggested that Tr1 did not play a discernable role in Dox-dependent redox cycling in vivo.

Ammonium sulfate fractionation of mouse liver cytosol provided further evidence for the existence of non-Cbr1 Dox-reductases. High levels of Doxol forming activity were found in fractions containing little or no Cbr1. Biochemical fractionation could be a viable method to purify and identify the remaining unknown Dox-reductases. Once identified, the relative contributions of these enzymes to Doxol formation could be assessed using the immunoclearing method described herein.

In summary, the major contributions of this study are validating a broadly useful methodology for quantifying the contribution of specific enzymes to the total activity observed in complex cell extracts, demonstrating that Cbr1 is responsible for 25% of the Doxol-forming activity in mouse liver cytosol, and showing that Cbr3 and Tr1 play no significant role in Dox metabolism in vivo, despite their ability to
metabolize Dox *in vitro*. More work is needed to identify the remaining Dox-reducing activities, but this study outlines a potential framework which could be used to purify and identify these unknown enzymes and subsequently assess their contribution to Doxol formation.
BIBLIOGRAPHY


Appendix A: Dox-dependent NADPH oxidation kinetics data

Appendix Figure A.1. Kinetic analysis of Dox-dependent NADPH oxidation by Cbr1
Purified recombinant Cbr1 (1 µM) was incubated with 200 µM NADPH and specified concentrations of Dox, and the change in A340 was monitored for 1 at 37°C. Kinetic parameters were calculated via (A) Non-linear least squares analysis, (B) Lineweaver-burk plot, (C) Eadie-Hoffstee plot, and (D) Hanes-Woolf plot.
Appendix Figure A.2. Kinetic analysis of Dox-dependent NADPH oxidation by Cbr3
Purified recombinant Cbr3 (1.9 μM) was incubated with 200 μM NADPH and specified concentrations of Dox, and the change in A$_{340}$ was monitored for 1 at 37°C. Kinetic parameters were calculated via (A) Non-linear least squares analysis, (B) Lineweaver-burk plot, (C) Eadie-Hoffstee plot, and (D) Hanes-Woolf plot.
Appendix Figure A.3. Kinetic analysis of Dox-dependent NADPH oxidation by Tr1

Purified recombinant Tr1 (1.9 µM) was incubated with 200 µM NADPH and specified concentrations of Dox, and the change in A$_{340}$ was monitored for 1 h at 37°C. Kinetic parameters were calculated via (A) Non-linear least squares analysis, (B) Lineweaver-burk plot, (C) Eadie-Hoffstee plot, and (D) Hanes-Woolf plot.
Appendix B: NADPH-dependent Doxol formation kinetics data

Appendix Figure B.1. Kinetic analysis of NADPH-dependent Doxol formation by Cbr1
Purified recombinant Cbr1 (1 μM) was incubated with 200 μM NADPH and specified concentrations of Dox for 1h at 37°C, and the amount of Doxol produced was determined by LC-MS/MS. Kinetic parameters were calculated by (A) non-linear least squares analysis, (B) Lineweaver-burk plot, (C) Eadie-Hoffstee plot, and (D) Hanes-Woolf plot.
Appendix Figure B.2. Kinetic analysis of NADPH-dependent Doxol formation by Cbr3
Purified recombinant Cbr3 (1 µM) was incubated with 200 µM NADPH and specified concentrations of Dox for 1h at 37°C and the amount of Doxol produced was determined by LC-MS/MS. Kinetic parameters were calculated via (A) non-linear least squares analysis, (B) Lineweaver-burk plot, (C) Eadie-Hoffstee plot, and (D) Hanes-Woolf plot.
Appendix Figure B.3. Kinetic analysis of NADPH-dependent Doxol formation by mouse liver cytosol

Mouse liver cytosol (0.55 µg protein/µl) was incubated with 200 µM NADPH and specified concentrations of Dox for 1h at 37°C and the amount of Doxol produced was determined by LC-MS/MS. Kinetic parameters were calculated using (A) non-linear least squares analysis, (B) Lineweaver-burk plot, (C) Eadie-Hoffstee plot, and (D) Hanes-Woolf plot.
Appendix C: Abbreviations

Akr: aldo-keto reductase
Cbr1: carbonyl reductase 1
Cbr3: carbonyl reductase 3
DNA: deoxyribonucleic acid
Dox: doxorubicin
Doxol: doxorubicinol
DTT: dithiothreitol
EDTA: ethylenediaminetetraacetic acid
EGTA: ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
gcml: γ-glutamyl-cysteine ligase modulatory subunit (gene)
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP: horseradish peroxidase
Hydroxy-PP: 4-amino-1-tert-butyl-3-(2-hydroxyphenyl)pyrazolo[3,4-d]pyrimidine
Hydroxy-PP-Me: 3-(7-isopropyl-4-(methylamino)-7H-pyrrolo[2,3-d]pyrimidin-5yl)phenol
IACUC: Institutional Animal Care and Use Committee
IgG: immunoglobulin G
LARC: Laboratory Animal Resources Center
LC-MS/MS: liquid chromatography with tandem mass spectrometry
MOPS: 3-(N-morpholino)propanesulfonic acid
NADPH: nicotinamide adenine dinucleotide phosphate
NFDM: non-fat dried milk
Nrf2: nuclear factor erythroid 2-related factor 2
PBS: phosphate-buffered saline
RNA: ribonucleic acid
ROS: reactive oxygen species
SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TopoII: topoisomerase II
TBST: tris-buffered saline with Tween
Tr1: thioredoxin reductase 1
Tris: tris(hydroxymethyl)aminomethane
Trx1: thioredoxin
txnrd1: thioredoxin reductase (gene)
wt: wild type